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14. ABSTRACT

This is an annual report in no cost extension period. In this period we have identified several compounds via virtual screening. These compounds include small molecules – transition state analogues and benzimidazoles. We have determined the crystal structure of BoNT/E with the substrate tetra peptide containing the scissile bond.

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Table of Contents

Page

Introduction……...4

Body……4

Key Research Accomplishments……7

Reportable Outcomes……7

Conclusions……..7

References……7

Appendix……

Structural Studies on Intact *Clostridium botulinum* Neurotoxins Complexed with Inhibitors Leading to Drug Design
Annual Report for the Period ending January 2008

Introduction

The overall major goal of this project is to design small molecule and peptidic inhibitors for botulinum neurotoxins. Botulinum neurotoxin act via a four step process: 1. Binding to neuronal cell; 2. Internalization into vesicles; 3. Translocation through endosomal membrane into cytoplasm, and 4. catalytic activity exerted on one of the three proteins forming SNARE complex required for docking and fusion to target cells for neurotransmitter release. The three structural domains are responsible for these steps and blocking any one of the steps will provide a counter measure to thwart toxicity. In our proposal we identified two targets – binding domain and catalytic domain. Our major effort is to design small molecules or peptides capable of blocking the binding of gangliosides to the binding domain or blocking the active site of catalytic domain to stop the catalytic activity. The conventional drug design is based on identifying a lead molecule and then determining the structures of lead molecules in complex with the toxin or the relevant target and then modifying the inhibitor chemically for better inhibition in an iterative manner. A two-pronged approach is being used with regard to catalytic domain. We use virtual screening of small molecule libraries to identify potential lead molecules. In the second approach, we use the substrate information to design structure and substrate based inhibitors. The general approach is to study the crystal structure of the toxin in complex with a potential inhibitor via x-ray crystallography and then analyze the interactions between the inhibitor and the protein.

Body

(1) Studies with C. neurotoxin catalytic domains

Since our method is structure based drug design, the first step in our project is to obtain high resolution crystal structures of relevant domains of botulinum neurotoxins.

We have successfully cloned all seven serotypes of botulinum neurotoxins. In this project period we tried to determine the optimal length of light chains required for catalytic activity of each serotype which helps in obtaining high quality crystals without compromising the catalytic activity. Such a work has been reported earlier for BoNT/A by Barbeiri's group. Following a similar logic we identified optimal lengths for BoNT/B, C, D, E, F and G. while maintaining the catalytic activity. The structure determinations were done to understand the mode of action of all serotypes which have different substrates. The study showed that the active site geometry has a common pattern and it may be possible to develop a common inhibitor to all of them. In this regard we have determined the structures of light chains of BoNT/A, BoNT/C (partly in collaboration), BoNT/B, BoNT/D (unpublished), BoNT/E, BoNT/F and tetanus toxin.

In the course of this work we found that by trimming the C terminal region of the catalytic domains, we could get better quality crystals and in turn higher resolution structures. As always, high resolution structures are better suited for drug discovery program.

(2) Virtual Screening of small molecule database:

Initially, we considered ZINC database (Zinc Is Not Commercial) for virtual screening. Though it contained more than 72,000 compounds when we started our project, it has grown to more than a million compounds. Since this is a computer intensive program, we used a different strategy in this project period. It has been established that benzimidazole compounds are good zinc chealators and since botulinum neurotoxin catalytic domains are zinc endopeptidases we first selected a subset of compounds containing benzimidazole moieties. We pulled out nearly 9000 compound containing both substituted and non-substituted benzimidazole. We used DOCK 6.1 to assess the docked conformations and to obtain binding energies. The compounds successfully docked were sorted out according to their energies and we took the top 40 compounds for further study. We are in the process of analyzing their efficacies by in vitro HPLC assay using suitable substrate peptides. We have so far identified three compounds which may be potential lead compounds. This work is in progress.

A second set of compounds containing sulfonic or phosphoric acid moieties which will mimic transition state of catalytic activity was selected. About top 40 compounds have been selected for in vitro inhibition studies.

In addition to ZINC database, we have now included NCI chemical database and have identified about 40 compounds from the diversity list. In vitro analysis shows that three of them are very good lead compounds. Our aim is to identify other homologous compounds and chemically modify them for better inhibition.

(3) Design of substrate based peptides:

The substrates for botulinum neurotoxins are large poly peptides. SNAP-25, the substrate for BoNT/A, C, and E is a 25 kDa molecule. Though it has been proved that the toxin recognizes the substrate at two exosites in addition to the active sites, we concentrated on the active sites. Initially, we purchased tetra peptides representing P1-P1'-P2'-P3' and determined the crystal structures of these peptides with BoNT/E light chain. For the first time we have shown how the substrate interacts with the toxin at the active site. We are preparing a manuscript for publication. Though this is not a very efficient inhibitor, it has given us a starting point to design peptidic inhibitors by modifying the substrate peptide. Similar work is carried out for other serotypes.

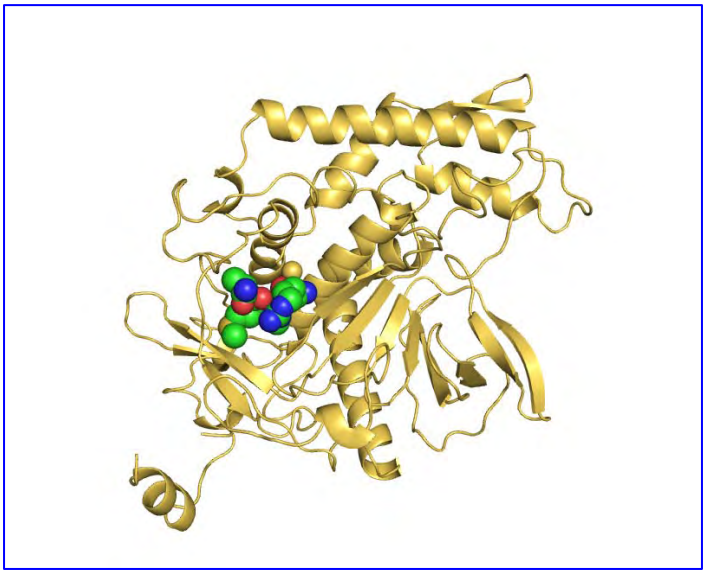

Figure 1. Ribbons representation of BoNT/E catalytic domain with substrate tetra peptide (in sphere model)

Key Research Accomplishments

- Crystal structure of BoNT/A and C catalytic domain have been determined helping us to understand the differences in substrate specificity. A manuscript describing the structure is being submitted for publication.
- A subset of small molecules that may inhibit the catalytic activity of BoNT/E has been identified and the work is continuing.
- Crystal structure of BoNT/E light chain in complex with substrate tetrapeptide has been determined.

Reportable outcomes

S. Swaminathan (PI) was invited to contribute a chapter in a book titled "Botulinum and other neurotoxins: Translating science into therapeutic applications".

1. S. Swaminathan. Molecular Structures and Functional Relationships of Botulinum Neurotoxins. In, *"Botulinum and other neurotoxins: Translating science into therapeutic applications"*. Ed. J. Jankovic, A. Albanese, M. Zouhair Atassi, J.O. Dolly, M. Hallett and N.H. Mayer. Elsevier, Inc. In press.

Conclusions

In our studies we have shown that better diffracting crystal to produce higher resolution structures could be obtained by truncating the C-terminal region without compromising the catalytic efficiency. Crystal structure of BoNT/E catalytic domain in complex with a substrate peptide has shown the interactions of the active site residues with the substrate peptide. We have identified a subset of small molecules as potential inhibitors for BoNT/E via virtual screening.

Plans for the next year:

We will complete the virtual screening of BoNT/E with Dock Autodock programs. Crystallize and determine the structure of BoNT/A binding domain. Also structural work on BoNT/B-inhibitor complex will be continued.

Personnel in the Project

1. S. Swaminathan (PI)	Scientist	30% effort
2. Mike Silberstein	Research Associate	75% effort